Eco. Env. & Cons. 26 (2) : 2020; pp. (663-668)
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ISSN 0971–765X

Oxidative stress effects of zinc oxide nanoparticles on fresh water microalga *Haematococcus pluvialis*

***Sinouvassane Djearamane[1], Ling Shing Wong[2], Yang Mooi Lim[3] and Poh Foong Lee[4]**

[1]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, 31900 Malaysia
[2]Life Science Division, Faculty of Health and Life Sciences, INTI International University, Nilai, 71800 Malaysia
[3]Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Bandar Sungai Long, 43000 Malaysia
[4]Department of Mechatronics & Biomedical Engineering, Lee Kong Chian Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, Bandar Sungai Long, 43000 Malaysia

(Received 5 October, 2019; accepted 28 December, 2019)

ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are enormously utilized in the consumer products in recent days due to their unique physio-chemical properties. Such substantial application in commercial products has resulted in the huge release of ZnO NPs into the environment, especially aquatic environment. The presence of ZnO NPs in the aquatic environment might result in the growth inhibition of aquatic organisms through induction of oxidative stress by the excessive release of reactive oxygen species (ROS) and the subsequent lipid peroxidation. Thus, the present study has investigated the oxidative stress effects of ZnO NPs in fresh water microalga *Haematococcus pluvialis*. The microalgal cells were exposed to the increasing concentrations of ZnO NPs from 10 to 200 mg/L over a period of 96 h at an interval of 24 h to explore the dose- and time-dependent oxidative stress effects of ZnO NPs in algal cells. The study results revealed a typical concentration- and time-dependent increase in reactive oxygen species and lipid peroxidation levels. Hence, the present study suggest the potential of microalga *H. pluvialis* to be used as a bio-indicator of ZnO NPs in aquatic environment due to the profound sensitivity of *H. pluvialis* towards ZnO NPs toxicity.

Key words: *Zinc oxide nanoparticles, Haematococcs pluvialis, Oxidative stress, ROS, Lipid peroxidation*

Introduction

The rising trend in the large-scale commercial production and use of ZnO NPs may result in an unintentional or intentional release of these particles from the industrial wastes into the aquatic environment (Baker *et al.,* 2014; Wang *et al.,* 2016; Djearamane *et al.,* 2016). Notably, the environmental contamination of ZnO NPs posing great threat to the aquatic organisms (Gottschalk *et al.,* 2009). The aquatic organisms including marine phytoplankton exhibit a wide range of sensitivity to dissolved zinc, though it is considered as an essential nutrient (Miller *et al.,* 2010). The adverse effects of ZnO NPs evidently need to be assessed on various aquatic organisms as an integral part of environmental risk assessment (Hazeem *et al.,* 2016) and there arises a need for better understanding and assessment of ecotoxicity of NPs to the key ecosystem organisms such as algae, plants, and fungi which are continu-

ously being exposed to these nanomaterials (Srivastava *et al.,* 2015). Alga is an organism that is sensitive to metallic contaminants when compared to fish and invertebrates (Zhou *et al.,* 2014). Therefore, investigating the oxidative stress effects of ZnO NPs on microalgae is essential as the induction of oxidative stress is the major cause for algal cell death. Hence, the present study tested the oxidative stress effects of ZnO NPs on fresh water green microalga *H. pluvialis*. The results of the present study might be useful for the application of microalgae as the potential bioindicator to monitor water contamination and aquatic toxicity of ZnO NPs.

Materials and Methods

Primary Characterization of ZnO NPs

Nano-zinc oxide powder with the specified particle size of <100 nm was procured from Sigma-Aldrich. X-ray diffractometer (Lab X, SHIMADZU, XRD-6000, Japan), operated at an angle of 2è with 40 volts and 30 mA current was applied to investigate the crystalline structure and size of the nanomaterial.

Cultivation of Microalgae

The fresh water microalga *H. pluvialis* stock cultures was procured from UTEX1926 (University of Texas Culture Collection, Austin, TX, USA). *H. pluvialis* was cultivated in Bold Basal Medium (BBM) which composed of $NaNO_3$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.025; $MgSO_4 \cdot 7H_2O$, 0.075; $NaHCO_3$, 0.0126; K_2HPO_4, 0.075; KH_2PO_4, 0.175; NaCl, 0.025; $Na_2EDTA \cdot 2H_2O$, 0.05; KOH, 0.031; FeSO4·7H2O, 0.005; $FeCl_3 \cdot 6H2O$, 0.00315; H_3BO_3, 0.01142; $ZnSO_4 \cdot 7H_2O$,0.00882; $MnCl_2 \cdot 4H_2O$, 0.00144; MoO_3, 0.00071; $Na_2MoO_4 \cdot 2H2O$, 0.000006; $CuSO_4.5H_2O$, 0.00157; $Co(NO_3)_2 \cdot 6H_2O$, 0.00049; $CoCl_2 6H_2O$. 0.00001 (Menezes *et al.,* 2016). The algal cultures were maintained in Erlenmeyer flasks under around 1200 lux illumination using white cool fluorescent lamp with 16 h light and 8 h dark conditions at room temperature (21- 23 °C).

Exposure of Microalgae to ZnO NPs

A 200 mL stock solution of 400 mg/L ZnO NPs was prepared by adding 80 mg of ZnO NPs powder in 200 mL of BBM medium. The stock solution was sonicated for 30 min at 40 kHz to prepare the homogenous solution of nanoparticles. The five testing concentrations of nanoparticles were prepared at 10, 50, 100, 150 and 200 mg/L by diluting the stock solution with the culture medium. *H. pluvialis* cells from 4th day culture, with an initial cell density of 1 x 10^5cells/mL, were subjected to the exposure with 10, 50, 100, 150 and 200 mg/L of ZnO NPs. 50 mL of algal cultures were mixed with 50 mL of the respective concentrations of ZnO NPs in 250 ml Erlenmeyer flask for a period of 96 h along with the control that was devoid of NPs. The ZnO NPs treated algal cells together with control cells were subjected for the oxidative stress assessment at 24, 48, 72 and 96 h respectively.

Oxidative Stress Assessment

Detection of Intracellular ROS in Algal Cells Treated with ZnO NPs

The production of intracellular reactive oxygen species (ROS) was investigated using fluorescent molecular probe 2',7'-dichlorodihydro fluorescein diacetate (H_2DCF-DA) (Invitrogen, Molecular Probes Inc., U.S.A.). For ROS measurement, a 0.5 mL of particle-exposed and untreated algal cell suspensions were washed twice in 1X PBS to remove the unbound NPs. Then 5 µL of 1mM dye was added to 1.0 mL of washed cell suspension to provide the final working concentration of 5 µM dye and incubated in dark for 30 min at room temperature (21- 23 °C). Then the stained cells were washed with 1X PBS to remove the unreacted dye and then the washed cells were re-suspended in 1X PBS. The green fluorescence emission from the algal cells was quantified using fluorescent microplate reader (TECAN, Infinite M200PRO, Switzerland) at an excitation / emission wavelength of 485/ 530. The percentage increase in ROS production was determined by comparing with control. The fluorescent microscopic examination (Nikon ECLIPSE 90i, United Kingdom) of H_2DCF-DA stained algal cells was carried out to further confirm the intracellular production of ROS in the algal cells that were exposed to ZnO NPs. In addition, the experiment included both negative control which contained the algal cells only and positive control which contained the algal cells treated with 5 % (v/v) hydrogen peroxide for 20 min.

Determination of Lipid Peroxidation in Algal Cells Treated with ZnO NPs

The level of lipid peroxidation (LPO) was assessed

through the measurement of Malondialdehyde (MDA), which is a well-known biomarker of LPO using BODIPY 581/591C11 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a-,4a-diaza-s-indacene-3-undecanoic acid-Invitrogen Molecular Probe, USA). BODIPY is a fluorescent fatty acid analog with fluorescent properties, which emits green fluorescence after the oxidation induced by the excessive production of intracellular ROS. After 24, 48, 72 and 96 h of treatment with NPs, 0.5 mL of particle-exposed and untreated algal cell suspensions were centrifuged at 5000 rpm for 10 min to remove the unbound NPs. The cells were washed twice with 1X PBS, followed immediately by addition of 5µL of 1mM dye to 1.0 mL of washed cell suspension to reach 5 µM of dye and incubated in dark for 30 min at room temperature (21- 23 °C). Then the stained cells were washed with 1X PBS to remove the unreacted dye and re-suspended in 1X PBS. The green fluorescence emitted from the algal cells was quantified by florescent microplate reader (TECAN, Infinite M200PRO, Switzerland) at excitation/emission wavelength of 485/530. Increase in lipid peroxidation level was determined by comparing with control. The experiment included both negative control which contained the algal cells only and positive control which contained the algal cells treated with 5 mM hydrogen peroxide for 30 min.

Statistical Analysis

The experiments were performed in triplicates (n=3) and the calculated results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons (SPSS version 22) was carried out for analyzing the significant level. The level of significance was accepted at p value < 0.05. A significant difference at p< 0.05 between the control (0 mg/L of ZnO NPs) and the tested concentrations (10, 50, 100, 150 and 200 mg/L of ZnO NPs) at the specific time period (24, 48, 72 and 96 h) are denoted with the symbol*.

Results

Characterization of ZnO NPs

The X-ray diffractometer (XRD) spectrum of nano-zinc oxide powder displayed the strongest diffraction peaks at 31.7°, 34.36° and 36.19° which are identical to the characteristic hexagonal wurtzite crystalline structure of ZnO NPs (Ramesh *et al.*, 2015). In addition, the XRD spectrum of ZnO NPs was used to further confirm the size of ZnO NPs using Scherrer's equation and the size was estimated to be at the range of 40 - 47 nm with a mean particle size of 43 nm.

Oxidative Stress Assessment

Determination of Reactive Oxygen Species

The toxic effects of ZnO NPs on microalga *H. pluvialis* induced a significant (p< 0.05) increase in in-vivo production of reactive oxygen species for all the tested concentrations of ZnO NPs (10 to 200 mg/L) from 24 h of exposure. A maximum ROS production was noticed at 96 h with the reported values of 451.5 ± 38.6, 674.1 ± 48.3, 836.9 ± 14, 894.9 ± 54 and 973 ± 75.2 % from *H. pluvialis* cells (Fig. 1) for 10, 50, 100 ,150 and 200 mg/L of ZnO NPs, respectively. The exposure of nano-zinc oxide material on *H. pluvialis* demonstrated a characteristic dose- and time-dependent increase in ROS production.

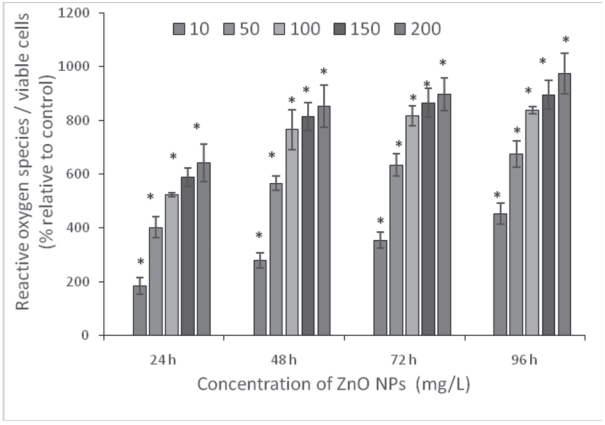

Fig. 1. Percentage increase in reactive oxygen species from *H. pluvialis* upon treatment with ZnO NPs.

Further, the intracellular production of reactive oxygen species was demonstrated by the green fluorescence of dichlorofluorescein diacetate in *H. pluvialis* cells treated with ZnO NPs. The fluorescent microscopic examination of H_2DCF-DA dye stained control cells showed red fluorescence. Whereas, the test cells treated with ZnO NPs displayed green fluorescence due to the cleavage of the fluorescent probe by the presence of excessive intracellular ROS that were produced through the oxidative stress resulted from the treatment of zinc oxide nanomaterial (Fig. 2).

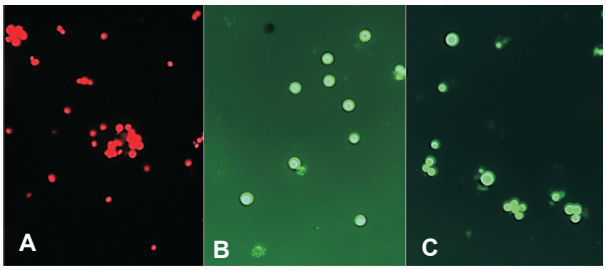

Fig. 2. Fluorescence images showing the DCF green fluorescence of algal cells due to oxidative stress. (A) Negative control - *H. pluvialis* without NPs treatment (red florescence); (B) Positive control- *H. pluvialis* treated with hydrogen peroxide (5% v/v) for 20 min (green fluorescence); (C) *H. pluvialis* treated with ZnO NPs (200 mg/L) at 96 h (green fluorescence).

In vivo production of ROS was demonstrated in algal cells exposed to ZnO NPs through the emission of green fluorescence. Similar findings were documented by Bhuvaneshwari *et al.,* (2015) who reported a concentration dependent increase in ROS production in freshwater alga *S. obliquus* with the reported values of 125.5% for 1 mg/L of ZnO NPs at 72 h. In addition, authors demonstrated the intracellular production of ROS by DCF fluorescence from the algal cells treated with nanoscale zinc oxide particles. Dalai *et al.,* (2014) reported the induction of dose dependent ROS production by 147.43, 161.24 and 190.2% respectively during the treatment with 0.05, 0.5 and 1 mg/L of mixed titanium dioxide NPs (TiO_2NPs) and aluminaNPs on *S. obliquus*at 72 h. A study by Oukarroum *et al.,* (2012) demonstrated a does dependent enhanced ROS formation in *D. tertiolecta* over *C. vulgaris* upon 24 h treatment with 1 - 10 mg/L of silver nanaoparticles (Ag NPs). Besides microalgae, Zhang *et al.,* (2016) and Dalai *et al.,* (2012) showed the intracellular production of ROS in yeast *S. cerevisiae* with ZnO NPs (2 mg/L) at 48 h and in freshwater bacteria with 2 h treatment of TiO_2 NPs (1 mg/L) respectively.

Determination of Lipid Peroxidation Level

Increase in LPO level by the treatment of ZnO NPs on *H. pluvialis* showed a similar pattern with ROS production. Results showed a significant (p< 0.05%) increase in LPO level from 24 h for all the tested concentrations of ZnO NPs. The highest level of LPO was reported to be 109.5 ± 13.7, 214.9 ± 19, 293.1 ± 26.1, 312.1 ± 29.2 and 323.9 ± 21.6% at 96 h for 10, 50, 100, 150 and 200 mg/L, respectively (Fig. 3). Similar to ROS production, results exhibited a distinctive dose- and time-dependent increase in LPO level by the treatment of nanoscale zinc oxide particles on *H. pluvialis.*

The quantification of MDA can be used to evaluate the status of LPO (Melegari *et al.,* 2012). The previous investigations by Choudhary *et al.,* (2007) and Deniz *et al.,* (2011) measured MDA level as a measure of the degree of lipid peroxidation and showed that the level of MDA increased with the increasing concentrations of trace metals including copper and zinc on *Spirulina* cells. In addition, the authors also conveyed that the LPO was linked to the concentration dependent free radical formation in *Spirulina* sp. A study by Li *et al.,* (2008) demonstrated a linear relationship between the loss in photosynthetic activity and increase in MDA level in *Spirulina* cells when exposed to intense light (250 µmol m^{-2} s^{-1}) indicating the photooxidative stress of photosynthetic apparatus (thylakoid membranes), that in turn resulted in reduction and or impairment of photosynthesis. Djearamane *et al.,* (2018) demonstrated the destruction of photosynthetic apparatus and the subsequent reduction in algal cell viability and biomass due to the treatment of ZnO NPs on *S. platensis*.

Similarly, Oukarruom *et al.* (2012) demonstrated a dose and time dependent increase in ROS and LPO, with higher level of ROS and LPO in *C. vulgaris* compared to the cell wall lacking microalga *D. tertiolecta* when treated with Ag NPs. The authors also reported a positive relationship between ROS production and LPO. Besides microalgae, toxicity of ZnO NPs has been reported to cause oxidative stress through a dose dependent increase in MDA

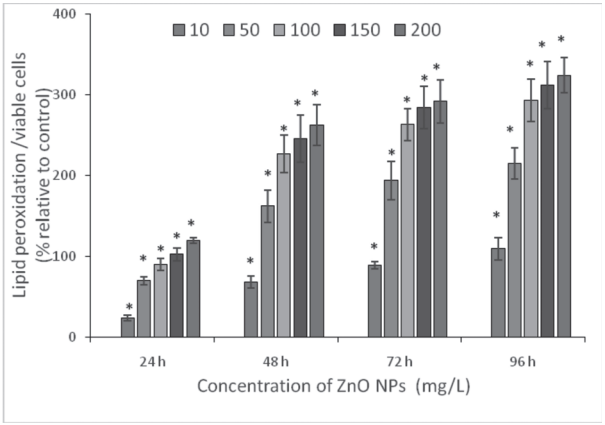

Fig. 3. Percentage increase in lipid peroxidation level on *H. pluvialis* upon treatment with ZnO NPs.

formation as a result of LPO on human hepatocytes and human embryonic kidney cells treated with 50, 75 and 100 mg/L of nanoscale ZnO particles at 24 h (Guan *et al.,* 2012). Apart from the measurement of ROS and LPO to assess the oxidative stress, Comotto *et al.,* (2014) used quantification of phenolic compounds to assess the oxidative stress caused by the treatment of TiO_2 on *S. platensis* and *H. pluvialis*. The phenolic compounds are the group of antioxidants that are produced to scavenger the oxidative stress. Exposure of pure anatase TiO_2 to both microalgae resulted in the increased release of extracellular phenolic compounds to scavenger the free radicals produced by oxidative stress. Further, Choudhary *et al.,* (2007) reported a dose dependent increase in anti-oxidant enzyme superoxide dismutase level as the indicator of oxidative stress induced by heavy metals such as lead, zinc and copper in *S. platensis*. Additionally, the exposure of *C. vulgaris* to ZnO NPs resulted in algal cell death and loss in algal biomass due to the reduction in chlorophyll content of algal cells by the toxicity of ZnO NPs (Djearamane *et al.,* 2019a; Djearamane *et al.,* 2019b).

Metals ions are proven to induce oxidative stress through the excessive production and accumulation of hydrogen peroxide and the resulting lipid peroxidation (Choudhary *et al.,* 2007). ROS reacts on cell membrane and oxidizes the membrane proteins, cholesterol and more predominantly the poly unsaturated fatty acids through a process called lipid peroxidation (Dingjan *et al.,* 2016). Lipid peroxidation spontaneously results in damaging the cell membrane that enables the entry of excessive NPs into the cells and leading to disturbance in the vital functions of cellular organelles (De Jesus and Kapila, 2014; Gallo *et al.,* 2016). As a consequence, disintegration of cell membrane and the leakage of intracellular contents result in death of algal cells (Valko *et al.,* 2006; Dingjan *et al.,* 2016). The present study demonstrated the induction of oxidative stress in *H. pluvialis* by the toxicity of ZnO NPs. The study findings suggest the possible reason behind the significant reduction in cell viability and algal biomass along with destruction of thylakoids in *H. pluvialis* by the treatment of ZnO NPs that were reported in our earlier study (Djearamane *et al.,* 2019c).

Conclusion

The exposure of fresh water microalgae *H. pluvialis* to ZnO NPs resulted in a characteristic dose and time dependent increase in ROS and lipid peroxidation levels due to the oxidative stress induced by the toxic effect of ZnO NPs. The induction of oxidative stress might have resulted in growth inhibition of algal cells through reduction in photosynthetic pigments. Because of the profound sensitivity of *H. pluvialis* to the exposure of ZnO NPs, this study recommends the microalga *H. pluvialis* to function as a bioindicator for the ecotoxicological assessment of ZnO NPs in the aquatic environment.

Acknowledgement

This work was supported by the Ministry of Education, Malaysia (Grant No. FRGS-1-2014-SG03-INTI-02-1), UniversitiTunku Abdul Rahman Research Fund, UTARRF 2017, (Vote No.: 6200/LJ3).

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